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TRANSMURAL AND SUBCELLULAR LOCALIZATION OF MONOAMINE OXIDASE AND CATECHOL-*O*-METHYL TRANSFERASE IN RABBIT AORTA*

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Abstract—A quantitative study of monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) has been made on homogenates of adult rabbit thoracic aorta. Most of the activity of both enzymes is located in the tunica media. The ratio of adventitia/media distribution of COMT is considerably less than the corresponding ratio for MAO. The specific activities of MAO and COMT change in parallel through the greater part of the transmural profile obtained on 24 μ cryostat sections. The specific activity of COMT drops significantly in sections that include endothelium and subendothelial media, where no decrease in MAO is noted. Subcellular distribution studies indicate the presence of MAO in mitochondrial and microsomal fractions, compared to the predominantly microsomal and cell sap localization of COMT.

THE INACTIVATION of catecholamines released from sympathetic nerve endings occurs through three major pathways. Perhaps the most significant is the uptake and rebinding of liberated catecholamine by the nerve terminal.[1] A proportion of the liberated catecholamine that escapes uptake diffuses through the interstitium and emerges from the tissue unchanged, while the remainder is inactivated through enzymic degradation.[2] *O*-methylation of *l*-norepinephrine by catechol-*O*-methyl transferase (COMT) is significant in this respect. A further route of inactivation is via the oxidative deamination of the monoamine by monoamine oxidase (MAO). Recent work has shown that MAO plays a minor role in the inactivation of liberated norepinephrine, but plays a more significant role in maintaining the equilibrium of the stored stable and labile norepinephrine pools.[3]

MAO is localized in mitochondria isolated from brain,[4,5] liver[6] or adrenal.[7] De Champlain *et al.*,[8] in a study of the subcellular localization of MAO in rat tissue found a significant fraction of the enzyme in the microsomes of the heart, vas deferens, and the salivary glands. Such studies suggest a variable subcellular distribution for MAO which in some organs may be associated with the norepinephrine storage particles themselves, present in the microsome fraction.

With the advent of sensitive radiometric and fluorometric techniques for the estimation of COMT and MAO, it became feasible to attempt detailed measurements of these enzymes in the blood vessel wall. Previous studies have indicated the presence of MAO[9–12] and COMT[13,14] in animal vascular tissue. Such studies became more

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important after pharmacologic observations[15] revealed a significant amount of *l*-norepinephrine was deaminated or *O*-methylated in the tunica media.

The purpose of this study was to establish the regional and transmural distribution of COMT and MAO in adult rabbit aorta with special reference to the relative distribution between and within adventitia and tunica media and their subcellular localization in the primary fractions.

MATERIALS AND METHODS

Reagent grade chemicals were used unless otherwise indicated. *l*-Norepinephrine HCl, *dl*-normetanephrine, homovanillic acid (mol. wt. 182·2), oxalacetic acid, NADH, tyramine hydrochloride (mol. wt. 174) and cytochrome c (equine heart) were obtained from California Biochemicals, Los Angeles, Calif. Hydrogen peroxide standards were prepared from a 30% stabilized solution (mol. wt. 34·02) and maintained in the dark at 2°. Sucrose used in differential and gradient centrifugation was ultrapure from Mann Research, New York. Iproniazid was purchased from Pfizer Laboratories, New York. Horseradish peroxidase (type III) was obtained from Sigma. Methyl [^{14}C]S-adenosylmethionine was purchased from Schwartz BioResearch.

Preparation of tissue. Adult rabbits, 2·5–3·0 kg, were killed by cervical dislocation and bled rapidly. The aorta (blood vessel) was removed on a glass rod and rinsed in cold 0·25 M sucrose–10 mM Tris HCl, pH 7·4. Excess fat and para-aortic tissue were removed in the cold room. The aorta was chopped after weighing and homogenized in a conical glass-glass Potter–Elvehjem homogenizer. The pestle was driven by a slip-ring rotor at a variable speed 10–160 rev/min for 5–8 min. Subcellular centrifugal fractions were obtained on a Spinco centrifuge after the manner of Verity and Bevan.[16] Homogenates were placed in 5-ml cellulose acetate tubes and centrifuged at 3000 *g* for 10 min, providing supernatant (S_1) and pellet (P_1) fractions. Such a scheme provided mitochondrial (P_2), intermediate (P_3), microsomal (P_4) and cell sap (S_4) fractions.

In separate experiments, the intermediate (P_3) and microsomal (P_4) pellets were pooled and resuspended in 0·35 M sucrose. Of this pooled fraction, 1·0 ml was placed on top of a two-step discontinuous sucrose gradient consisting of 2 ml 2 M sucrose and 2 ml of 1·4 M sucrose. This gradient was centrifuged at 45,000 rev/min for 1 hr, providing upper (a) and intermediate (b) bands.

The transmural activity of MAO and COMT was made on aorta slices prepared in a cryostat after the method of Bevan *et al.*[17] In this method, four 24-μ sections were pooled for biochemical analysis. The pooled sample was numbered with reference to the position of the adventitio-medial junction and homogenized in a glass-glass homogenizer in 1 ml of 0·1 M sodium phosphate buffer, pH 7·4. Aliquots were removed for enzyme and protein determination.

Enzyme assays. COMT activity was assayed according to Krakoff *et al.*[18] without modification. The [^{14}C]normetanephrine formed from *l*-norepinephrine and [^{14}C]S-adenosylmethionine was extracted into toluene–isoamyl alcohol (3:2). The same R_f value (0·35) as authentic *dl*-normetanephrine was obtained on cellulose thin-layer chromatography using butanol–acetic acid–water (4:2:1) as the solvent system. Units of activity are expressed as nanomoles of [^{14}C]normetanephrine formed per hour.

MAO was assayed fluorometrically.[19] The method is based on the formation of an intensely fluorescent product between homovanillic acid and the hydrogen peroxide released during the oxidative deamination of tyramine. The incubation system (total

volume, 2·5 ml at 37°) contained: 2 ml of 0·1 M sodium phosphate buffer, pH 7·8; 2·2 mM tyramine HCl; $5{\cdot}7 \times 10^{-4}$ M homovanillic acid; 0·04 mg horseradish peroxidase; and 50–100 μl homogenate or fraction. The system was incubated with intermittent shaking for 15 min. Enzyme reaction was stopped by rapid chilling at 2°. If necessary, the tubes were centrifuged in the cold for 4 min to remove tissue turbidity. Fluorescence readings were obtained in a Farrand spectrofluorometer at room temperature using excitation wavelength 315 mμ and emission wavelength 425 mμ. A linear internal calibration curve for each experiment was prepared by the addition of 10 nmoles H_2O_2 (1·0 nmole H_2O_2/μl) to the tissue blank cuvette. Enzyme activity is expressed as nanomoles of H_2O_2 formed per minute.

Malate dehydrogenase was measured by a modification of the method of Bergmeyer and Bernt.[20] Cytochrome c oxidase was assayed by the spectrophotometric method of Cooperstein and Lazarow.[21] Enzyme activity is expressed as ΔE_{350} per minute. Protein was determined by the method of Lowry *et al.*[22] Aliquots of homogenate or fraction (10–50 μl) were digested overnight at 37° in 2 ml of the alkaline tartrate–carbonate solution prior to addition of phenol solution. Crystalline bovine serum albumin (Pentex) was prepared as an internal standard.

RESULTS

Characterization of vascular MAO and COMT. The rate of formation of the homovanillic acid fluorophore was linear over 30 min with vascular tissue as source of MAO. The activity of COMT was linearly related to the quantity of aortic tissue. The limit of sensitivity was adjudged to be equivalent to the supernatant obtained from 0·05 to 0·1 mg of aorta homogenate in that it gave at least 50 counts/min over the tissue blanks with an 83 per cent counting efficiency. The activity of aortic COMT was inhibited 63 and 95 per cent by 10^{-4} and 10^{-3} g/ml of tropolone respectively. No significant difference in MAO or COMT activity of homogenates prepared from upper or lower thoracic aorta was observed. For instance, mean values of 13·7 (N = 3) and 15·2 (N = 4) nmoles H_2O_2 produced/min/100 mg wet weight of tissue, respectively, were obtained for MAO.

Furchgott *et al.*[23] found that iproniazid strongly inhibited MAO of rabbit aorta. We obtained 100 per cent inhibition of postnuclear supernatant activity at an effective concentration of 1·0 mM iproniazid. These characteristics attest to the validity of the assays as performed on homogenates and subcellular fractions of rabbit aorta.

Distribution of MAO and COMT between adventitia and tunica media. We have shown a significant loss of unchanged *l*-norepinephrine through the adventitia,[15] after electrical transmural stimulation of the neuronal plexus, known to be aggregated at the aortic adventitio-medial interface. Conversely, the liberated *l*-norepinephrine diffusing through the media is methylated or deaminated, or both. Such pharmacologic data may be explained by presupposing a significant difference in the concentration or distribution of MAO and COMT between adventitia and media.

To test this possibility, the tunica adventitia was separated from tunica media after the manner of Maxwell *et al.*[24] Table 1 summarizes the mean values for MAO and COMT in the adventitia and media (see also Fig. 2). Of note, is the difference between the adventitial/medial ratio of MAO, compared with that of COMT, which emphasizes the relatively low activity of COMT in the adventitia.

TABLE 1. DISTRIBUTION OF MAO AND COMT ACTIVITIES IN ADVENTITIA AND TUNICA MEDIA OF ADULT RABBIT AORTA*

	Mean wet wt. (mg)	Enzyme activity/100 mg wet wt.	
		Monoamine oxidase	Catechol-*O*-methyl transferase
Adventitia	28·6 ± 4	5·5 ± 2·1	0·031 ± 0·00
Media	50·4 ± 5	20·6 ± 4·0	0·69 ± 0·02
Media/adventitia ratio		3·7	22·2

* Adventitia is split from thoracic aorta after the technique of Maxwell *et al.*[20] Values are means of five to eight animals ± S.E.M. MAO activity is expressed as nanomoles H_2O_2 produced per minute; COMT activity as nanomoles [^{14}C]normetanephrine per hour. The adventitial COMT activity gave counts per minute significantly greater than blank values ($P < 0·02$).

Content of MAO and COMT in subcellular fractions of rabbit thoracic aorta. The mean wet weight of the thoracic aorta used for the preparation of homogenates was 305·8 mg (N = 9). The specific activities of MAO and COMT were greatest in the microsomal (P_4) fractions (Table 2). This fraction accounted for approximately 10–15 per cent of the total activity of each enzyme. A significant postnuclear fractional partition of COMT was seen in the cell sap (46 per cent) with only 2 per cent in the mitochondrial (P_2) fraction, compared to 16 per cent of MAO in the mitochondrial fraction. These results suggest that COMT is predominantly localized in the cell sap with a significant component in the microsomes, while MAO is predominantly in the microsomal reticulum with a small component in the mitochondrial membrane. In this respect, the subcellular distribution of MAO in the aorta is similar to that of the vas deferens and heart.[8,25]

TABLE 2. TOTAL AND SPECIFIC ACTIVITIES OF MAO AND COMT IN SUBCELLULAR FRACTIONS OF ADULT RABBIT AORTA*

Fractions (*g*-min)	Protein (mg)	Monoamine oxidase (nmoles/min)	Catechol-*O*-methyl transferase (nmoles/hr)
S_1	49·2	144·8 ± 19 (2·84)	16·5 ± 3·8 (0·35)
P_1 (3×10^4)	118·8	33·6 ± 7 (0·28)	3·9 ± 0·8 (0·03)
P_2 ($1·2 \times 10^5$)	8·6	23·2 ± 5 (2·70)	0·37 ± 0·03 (0·05)
P_3 ($1·5 \times 10^6$)	7·5	33·0 ± 3 (4·44)	3·1 ± 0·6 (0·42)
P_4 (6×10^6)	3·1	32·1 ± 3 (10·3)	3·59 ± 0·5 (1·19)
S_4	27·2	32·0 ± 6 (1·18)	7·61 ± 0·9 (0·30)
Recovery†	91	84	89

* Fractionation of 0·25 M sucrose–10 mM Tris–HCl homogenates performed after the method of Verity and Bevan[12] (see Methods for definition of fractions). Individual experiments are normalized to gram equivalent wet weight of aorta. Values are means ± S.E.M. (n = four to six). Total activity represents activity per fraction; specific activity (in parentheses) is per milligram of protein.

† Recovery expressed as percentages of S_1 fraction.

Activities of COMT and MAO in the three postnuclear membrane fractions compared to the activity of cytochrome c *oxidase and NADH cytochrome* c *reductase.* The sheer forces used in obtaining adequate homogenization of rabbit aorta allow for the formation of membrane fragments from large organelles which may be distributed in the smaller particle fractions. In a previous study[16] on the subcellular distribution of adenosine triphosphatase (ATPase) activity in the rabbit aorta, three postnuclear membrane fractions were obtained and characterized with respect to selected marker enzymes. The same fractionation scheme was used herein with appropriate marker enzyme controls. A parallel comparison of the relative specific activities of COMT and MAO with NADH cytochrome c reductase (microsomal) and cytochrome c oxidase (mitochondrial) allows for an estimation of particle purification and enrichment of the individual enzymes in the particle fractions. Figure 1 represents in histogram form the relative specific activity profiles of the four enzymes. Virtually no COMT activity is present in the mitochondrial fraction (P_2). There is low but significant MAO in the mitochondrial fraction, which is paralleled in amount by mitochondrial NADH cytochrome c reductase. This latter rotenone-insensitive reductase is known to be associated principally with the microsomal fraction, but a small component is present in the outer mitochondrial membrane.[26] In the liver, MAO is also a part of the outer mitochondrial membrane.[26]

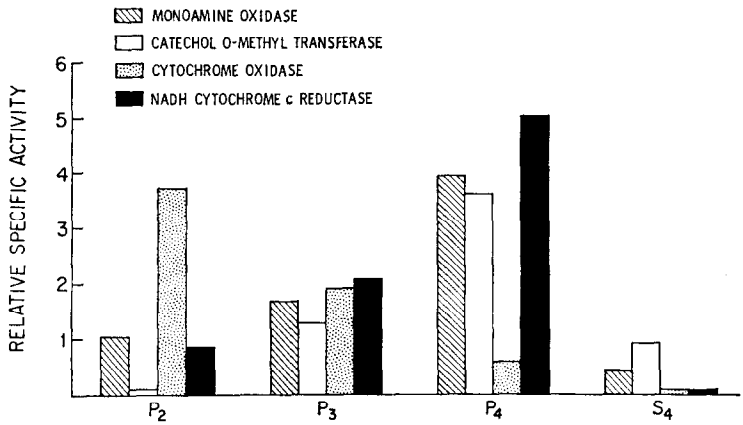

FIG. 1. Comparison of relative specific activities of MAO, COMT, cytochrome c oxidase and NADH cytochrome c reductase in the three postnuclear particulate fractions and cell sap. Relative specific activity for each fraction = per cent enzyme activity in each fraction/per cent fractional protein.

Discontinuous sucrose-density gradient centrifugation of postmitochondrial fraction. The demonstration of significant MAO activity in microsomal and mitochondrial fractions suggests that the microsomal activity may be an artifact of excessive homogenization and fragmentation of mitochondria with preferential sedimentation of the mitochondrial outer membrane fragment into the microsome fraction. Experiments were designed to examine the distribution of different membrane classes in the microsome fraction by virtue of changes in the specific activity of known enzyme markers. Aliquots of combined ($P_3 + P_4$) fractions were placed on a discontinuous sucrose gradient which, after centrifugation, provided two distinct bands, A and B

(see Methods). Table 3 summarizes the mean specific activities for MAO, cytochrome c oxidase and NADH cytochrome c reductase. Light microsomal membrane material will accumulate in band A, and is characterized by a 10-fold increase in NADH cytochrome c reductase activity over that found in band B. However, mitochondrial membrane fragments appear equally divided between A and B. The increased specific activity of MAO in band A provides confirmatory evidence for an association of MAO and NADH cytochrome c reductase in membranes derived from the fragmented smooth endoplasmic reticulum.

TABLE 3. SUCROSE DENSITY GRADIENT CENTRIFUGATION OF COMBINED ($P_3 + P_4$) FRACTIONS FROM AORTIC HOMOGENATES*

Bands	Protein (mg)	Monoamine oxidase	Cytochrome c oxidase	NADH cytochrome c reductase
$P_3 + P_4$	4·68	12·1 ± 2·2	0·14 ± 0·02	4·7 ± 0·8
A	1·51	22·7 ± 3·0	0·20 ± 0·02	25·5 ± 5·1
B	2·48	7·6 ± 2·3	0·19 ± 0·03	2·3 ± 0·9

* Distribution of protein, MAO, NADH cytochrome c reductase and cytochrome c oxidase in upper (A) and intermediate (B) bands. Values ± S.E.M. are means of three experiments. Specific activities are expressed as enzyme units per milligram of protein: MAO, nanomoles H_2O_2 per minute; cytochrome c oxidase, ΔE_{550} per minute; NADH cytochrome c reductase, ΔE_{550} per minute.

Transmural distribution of MAO and COMT. Su and Bevan[15] determined the distribution profile for the uptake of [^{3}H]norepinephrine across the aorta wall. This was obtained with a technique which involved rapid freezing in liquid nitrogen and sectioning in a cryostat of aorta tissue, parallel to the intimal surface. Using a similar sectioning technique, we have obtained a transmural distribution profile of the specific activities of MAO and COMT (Fig. 2). The standard error of the mean varied from

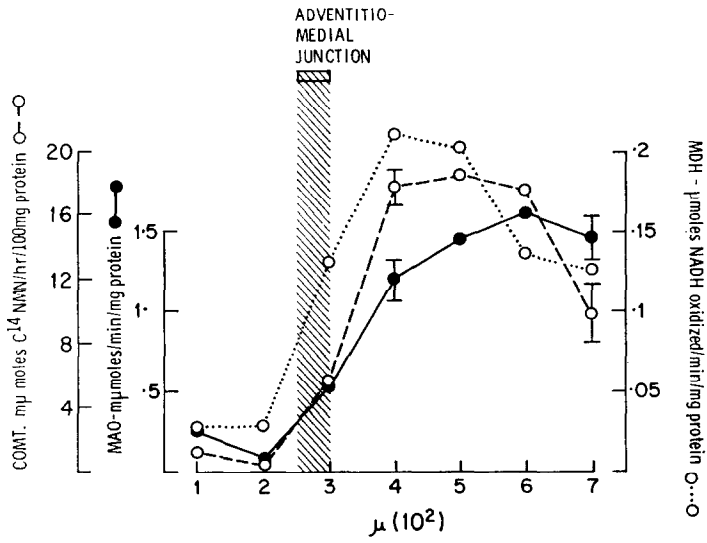

FIG. 2. Transmural profile of the specific activities of MAO, COMT and malate dehydrogenase. Enzyme activities and protein were determined on pooled, cryostat sections cut parallel to the elastic laminae. The position of the adventitio-medial junction was determined by visual inspection of the cryostat sections.

7 to 18 per cent in three to five experiments. COMT activities of both groups of adventitial slices were negligible in that the counts per minute were no more than 20 above the blank. However, one sample of adventitial slices (far left point in Fig. 2) gave a high value, thus increasing the mean value. The abrupt change in both enzyme activities at the adventitio-medial junction and the plateau of COMT and MAO activity across the thickness of the media (fractions 3–6) can be appreciated. An apparent drop in specific activity of COMT and mitochondrial malate dehydrogenase in the intimal-subintimal region contrasts to the continuing plateau high levels of MAO. Moreover, there is a small but apparent shift to the right in the peak activity of MAO compared to that of COMT. Such a distribution for MAO is suggestive of higher activity at the endothelial surface compared to COMT.

DISCUSSION

Any critical analysis of the role played by extraneuronal mechanisms of catecholamine inactivation demands a knowledge of the kinetic diffusion parameters of *l*-norepinephrine through vascular tissue compartments, site and distribution of the enzymatic degradation machinery in the vascular wall. Enzymatic degradation of *l*-norepinephrine released from neural stores may play a significant part in this disposition, as is evident from superfusion studies of the pulmonary artery during transmural stimulation.[15,27] A significant increase in deaminated catechol and noncatechol metabolites after stimulation indicated that part of the [^{3}H]NE released during nerve stimulation was enzymatically inactivated.[27]

Studies reported here quantitate and compare the distribution of MAO and COMT in the vessel wall and confirm the presence of MAO[10] and COMT,[13,14] but reveal an apparent difference in specific activity ratio between adventitia and media (COMT, 1:22; MAO, 1:4). It is not surprising that the enzymatic activity of the media is greater than that of adventitia, an observation demonstrated by histochemical procedures,[11,12] in the case of MAO. The small amount of COMT activity in the adventitial preparation may be accounted for by the few smooth muscle cell layers that have been histologically found to accompany it (J. A. Bevan and M. A. Verity, unpublished observations). Recent studies of Jacobowitz[28] have localized COMT and MAO in fibroblasts harvested from tissue culture constituting a further extraneuronal site for coordinated catecholamine metabolism. Thus, the relatively low adventitia/media activity ratio for COMT is consistent with the entirely intramuscular localization of this enzyme. However, in view of the comparatively larger adventitia/medial ratio for MAO and its well established presence within adrenergic nerves in other tissues, our results do not rule out an intraneuronal existence for MAO in the rabbit aorta. In two separate experiments on the subcellular distribution of MAO and COMT in adventitia-free media, no significant change in the distribution profile of the enzymes was observed in the primary centrifugation fractions. We must presume that an intraneuronal component of MAO is too small to be identified in such gross centrifugation procedures. While it is recognized that the neural apparatus is limited to the adventitio-medial junction, this structure would account for <1 per cent of the volume of this layer and would be masked by connective tissue elements in the transmural distribution studies of MAO and COMT. Conversely, assuming that MAO and COMT are located intracellularly and that smooth muscle accounts for approximately 50

per cent by volume of the media,[29] then the effective intracellular activity of the media should be increased considerably. In these studies, no allowance has been made for a possible contribution from the extracellular compartment to the cell sap activities. If such a contribution exists, then it will be minimal in these studies because the segments of aorta were rinsed numerous times in isotonic 0·25 M sucrose during the cleaning and preparation for homogenization.

De Champlain *et al.*[8] have suggested that the subcellular distribution of MAO is variable, depending on the tissue studied. In contradistinction are the views of Jarrott and Iversen,[25] who consider that MAO activities recovered in the microsomal fraction represent an artifact due to excessive mitochondrial disruption during homogenization. The mitochondrial localization of MAO has been thoroughly evaluated by Schnaitman *et al.*,[30] who favor an enzyme locus in the outer membrane. Evidence in favor of a true microsomal component in tissues, including aorta, is substantial. For instance: (1) Aortic NADH cytochrome c reductase and MAO reveal parallel changes in relative specific activity; in contrast is the inverse parallelism between MAO and cytochrome c oxidase. (2) Different tissues reveal very variable MAO distribution between mitochondrial and microsomal membrane fractions.[8,25] (3) The specific activity of mitochondrial succinic dehydrogenase and cytochrome c oxidase remains stable during heart growth[31] or isoproterenol-induced hypertropy of salivary gland,[32] whereas there is a marked increase in MAO activity. Such experiments suggest a dissociation between MAO and mitochondrial membrane enzyme reactivity.

Considerable COMT activity was found in the microsomal fraction. This contrasts with the view that COMT is localized in the cell sap only.[13] The low activity of COMT in the mitochondrial fraction indicates the insignificant cross-contamination of that fraction by microsomal membrane. Moreover, if the microsomal COMT component represents nonspecific adsorption of COMT from the cell sap, we would expect a significant adsorption of COMT to mitochondrial membrane also, unless it is proposed that microsomal adsorption occurs at a specific membrane receptor for COMT, a receptor missing in the mitochondrial fraction. It is likely, therefore, that the microsomal content of COMT is real, and cannot be accounted for by nonspecific membrane adsorption of COMT from the soluble cytoplasmic phase.

Although a considerable proportion of the activity of MAO and COMT is located in the microsomal fraction, the known heterogeneity of this fraction derived from aorta precludes any definite statement of the true intracellular localization. Evidence has been presented elsewhere[16] suggesting that components of the endoplasmic reticulum, plasmalemma and micropinocytic vesicles exist in the microsomal fraction, and membrane-bound MAO or COMT may be associated with any of these structurally distinct entities. Because of such reasons, it is difficult to postulate a role for the microsomal component of MAO in aortic media. De la Lande and Waterson[33] have proposed that inactivation of tyramine by MAO in the media is a factor contributing to the relatively low vasoconstrictor effect of intraluminal tyramine compared to extraluminal administration. Our finding of a significant, nonmitochondrial locus for MAO represented by sarcolemma or endoplasmic reticulum, or both suggests that tyramine (and other vasoactive amines) may be metabolized at extramitochondrial sites. It is conceivable that such sites include the sarcolemma and subsarcolemmal reticulum of vascular smooth muscle,[29] providing an external membrane site for metabolism of monoamines.

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